Interleukin 4 Expressed In Situ Selectively Alters Thymocyte Development

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Summary

Using a transgenic mouse model we show that increased intrathymic expression of interleukin 4 (IL-4) significantly perturbs the development of thymocytes. Transgenic double-positive ($CD4^+CD8^+$) thymocytes, which are present in dramatically reduced numbers, exhibit increased T cell receptor (TCR) expression and increased mobilization of calcium mediated by these receptors. In contrast, transgenic single-positive ($CD4^+CD8^-$ and $CD4^-CD8^+$) thymocytes and peripheral T cells exhibit decreased TCR-mediated calcium mobilization. The development of $CD4^-CD8^+$ thymocytes is significantly perturbed by IL-4 expressed in vivo; only peripheral $CD4^+$ T cells are found in significant numbers in transgenic mice, while $CD4^-CD8^+$ thymocytes are present in increased numbers, apparently because of their failure to emigrate to the periphery. In contrast to these selective effects on T cell development, no significant differences in the numbers of B cells or mast cells, or in the plasma levels of IgE and IgG1 are observed between transgenic and control mice. These observations suggest that IL-4 in vivo exerts its major effects locally rather than systemically, even when its expression is constitutively increased.

IL-4 is a cytokine secreted by T cells and mast cells which has pleotropic effects on a wide variety of hematopoietic cell types in vitro (1). Originally described as a growth factor for partially activated B cells (2), IL-4 has subsequently been shown to promote T cell and mast cell growth as well (3–5), and to have multiple effects on the differentiation of mature lymphocytes (1, 6–10). These IL-4–mediated effects include increased class II MHC surface expression and IgG1 and IgE production by B cells (6–8), enhanced generation of T cell–mediated cytotoxicity (9), and the induction of surface CD8 expression by $CD4^+$ T cell clones (10). Although these findings suggest that IL-4 regulates multiple aspects of antigen-specific immunity, the production of IgE by B-lineage cells is the only lymphocyte-mediated function which has clearly been shown to depend on the production of IL-4 in vivo (11, 12).

The expression of IL-4 mRNA by thymocytes in vivo during fetal ontogeny (13), and the secretion of IL-4 by fetal and adult $CD4^-CD8^-$ (double-negative) thymocytes after in vitro activation (14, 15), suggest that this cytokine may also have a role in normal T cell development. A number of in vitro studies have documented that IL-4 can either augment or inhibit the growth of isolated thymocytes, depending on the cell population studied, the stimulus used for cell activation, and which other cytokines are present (15–20). Studies of $CD4^-CD8^-$ thymocyte differentiation in vitro indicate that IL-4 may favor the development of double-negative or $CD8^+$ cells bearing γ/δ rather than α/β T cell receptors (16, 21). However, these studies do not satisfactorily address the function of IL-4 in an intact intrathymic context; it is likely that IL-4 and other cytokines significantly influence other components of the thymic microenvironment which provide essential signals regulating thymocyte growth, differentiation, and selection. Therefore, we have generated transgenic mice in which IL-4 is selectively expressed intrathymically. Such animals exhibit profound but selective disturbances in thymocyte development, including an accumulation of thymocytes bearing high levels of TCR-α/β and a $CD4^-CD8^+$ surface phenotype. Remarkably, intrathymic IL-4 expression does not alter the normal pattern of Ig isotypes in plasma or B cell or mast cell abundance. These results suggest that IL-4 can behave as a natural regulator of thymocyte maturation, but that its effects are exerted locally rather than systemically.

Materials and Methods

Preparation of the lck–IL-4 Construct. The *lck*–IL-4 construct (Fig. 1) was made by inserting a full-length murine IL-4 cDNA clone,

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0.7-kb BamH1 fragment (22), into the calf intestinal phosphatase-treated BamH1 site of an expression vector, p1017 (Chaffin, K. E., C. R. Beals, T. M. Wilkie, K. A. Forbush, M. I. Simon, and R. M. Perlmutter, manuscript submitted for publication) using standard techniques (23). In the final construct, the murine IL-4 cDNA segment was located 3′ to a 3.2-kb murine proximal *lck* promoter segment at +37 with respect to the transcription start site (24, 25), and 5′ to a 2.1-kb BamH1–EcoRI fragment of the human growth hormone (hGH)[1] gene (26). The IL-4 cDNA was the generous gift of Dr. E. Severinson (University of Stockholm, Stockholm, Sweden).

Generation of lck–IL-4 Transgenic Mice. A 6.0-kb NotI fragment containing the *lck*–IL-4 construct was purified by agarose gel electrophoresis onto DEAE–nitrocellulose paper (23) and diluted to 2 ng/ml in 10 mM Tris, 0.1 mM EDTA, pH 7.5. Aliquots of this preparation were injected into the pronuclei of C57BL/6J X DBA/2J F_2 hybrid mouse zygotes using standard techniques (27). Viable zygotes were then transferred into the oviducts of recipient pseudopregnant females. Mice bearing the construct were detected by hybridization of blotted tail DNA as previously described (25), using a ^{32}P-labeled 0.6-kb SmaI–EcoRI hGH fragment (26) as a probe. Transgene-positive lines were propagated by backcrossing founder animals with C57BL/6J mice. In all experiments, nontransgenic littermates served as age-matched, wild-type controls. Original strains of C57BL/6J and DBA/2J mice were obtained from The Jackson Laboratories (Bar Harbor, ME).

RNA Blots. Total RNA was isolated from tissue or cell preparations by the guanidinium-isothiocyanate/CsCl method (28). RNA was quantitated spectrophotometrically, electrophoresed in 1% agarose gels made in 2.2 M formaldehyde, and blotted as previously described (29). Hybridization and washing conditions were those previously described for ^{32}P-labeled DNA probes (30). The murine IL-4 probe, which consisted of a 0.6-kb BamH1–SspI cDNA fragment (22), was ^{32}P-labeled using the random hexamer priming method (31).

Cell Isolation and Stimulation. Single-cell suspensions of thymocytes and LN cells were prepared by finely mincing or teasing these tissues. To isolate spleen mononuclear cells, tissue was first disrupted with a fine-meshed sieve, and the mononuclear cell fraction was subsequently purified by Ficoll–Hypaque density gradient centrifugation (32). Bone marrow cells were collected by irrigation of femurs and humeri from euthanized mice (33). As necessary, contaminating red cells were removed by hypotonic NH_4Cl treatment (33).

IL-4 Assay. Thymocytes, splenocytes, or bone marrow cells (5×10^6/ml) from transgenic mice or control littermates were cultured in CT.4S medium (34) (RPMI 1640 [Mediatech, Washington, DC] with 10% FCS [Hyclone Laboratories, Logan, UT]), 1 mM Na pyruvate, 2 mM L-glutamine, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin for 24 h at 37°C in a humidified 5% CO_2 atmosphere. IL-4 activity was determined by the ability of supernatants from these cell cultures to support the incorporation of [^{3}H]thymidine by CT.4S cells (the kind gift of W. E. Paul, National Institutes of Health [NIH], Bethesda, MD), exactly as described (34). The concentration of IL-4 in samples was interpolated from a standard curve generated using purified recombinant murine IL-4. Based on this standard curve, the lower limit of detectability for IL-4 in the CT.4S assay was 10 pg/ml. An aliquot of each sample was preincubated with saturating concentrations (75 μg/ml) of purified 11B11 (anti-murine IL-4) mAb (35) for 45 min at 4°C before their addition to CT.4S cells, to confirm that [^{3}H]thymidine incorporation was IL-4 dependent. Purified recombinant murine IL-4 and 11B11 mAb were provided by Dr. K. Grabstein (Immunex Corp., Seattle, WA).

Immunofluorescent Flow Cytometry. The following preparations of anti-murine mAbs were used for flow cytometric analysis: 500A.2 (reference 36) (CD3) hybridoma supernatant; FITC-conjugated 145-2C11 (reference 37) (CD3); biotinylated H57-597 (reference 38) (pan-α/β TCR); PE-conjugated GK1.5 (reference 39) (CD4); biotinylated and FITC-conjugated 53-6.7 (reference 40) (CD8). 500A.2 was kindly provided by Dr. J. Allison (University of California, Berkeley, CA). 145-2C11 and H57-957 were gifts of Dr. A. Farr (University of Washington, Seattle, WA). Biotinylated-53-6.7 was generously provided by Dr. B. J. Fowlkes (NIH). The remaining mAbs were purchased from Becton Dickinson (Mountain View, CA). Single or two-color staining of cells was performed at 4°C in PBS/1% BSA/0.1% Na azide (staining buffer) with saturating concentrations of mAb as previously described (41). PE-conjugated streptavidin (Biomeda, Foster City, CA) was used for detection of biotinylated mAb. For single or two-color analysis, at least 3×10^3 lymphocytes per sample were analyzed using an Epics C instrument (Coulter Electronics Inc., Hialeah, FL) equipped with an argon laser. For three-color staining of CD3, CD4, and CD8, cells in staining buffer were sequentially incubated for 15 min at 4°C with 500A.2 hybridoma supernatant, FITC-conjugated goat anti-hamster IgG (mouse and rat IgG absorbed) (Caltag Laboratories, South San Francisco, CA), 10% normal rat serum, biotinylated 53-6.7, streptavidin–allophycocyanin (Biomeda), and PE-conjugated GK1.5. Cells were extensively washed between each step. Three-color analysis was performed on an Epics 753 instrument (Coulter Electronics Inc.) using argon and helium-neon lasers. At least 1×10^4 list mode–gated events were collected per sample. Data was analyzed using MDADS and REPROMAN software (Fred Hutchinson Cancer Research Center, Seattle, WA).

In Vivo Treatment with Anti–IL-4 mAb. Starting at 1 mo of age, transgenic mice or littermate controls were given three weekly injections by tail vein of either 10 mg of purified anti–IL-4 11B11 mAb (35) in 0.25–0.5 ml of PBS (pH 7.4) or PBS alone. Mice were sacrificed for analysis 1 wk after their final injection.

Ca^{2+} Analysis. Cells (10^7/ml in RPMI medium with 10% FCS) were loaded with 3 μg/ml of the acetoxymethyl ester of indo-1 (42) for 45 min at 37°C in darkness, washed, and then stained with saturating concentration of azide-free PE-conjugated CD4 and FITC-conjugated CD8 mAb (Becton Dickinson). After additional washing, cells were resuspended at 5×10^6/ml in RPMI medium. Cytoplasmic free (intracellular) calcium concentration ($[Ca^{2+}]_i$) was measured in individual cells by cytofluorometrically determining their ratio of violet to blue emissions as previously described (43); PE and FITC fluorescence were also simultaneously determined. Measurements were performed at 37°C at a rate of ~1,000 cells/s using a Cytofluorograph 50H with a model 2150 computer (Ortho Diagnostic Systems, Westwood, MA) as previously described (43, 44). When baseline $[Ca^{2+}]_i$ was established after 2 min of cell flow, azide-free purified anti-CD3 mAb, 145-2C11 (37), or Con A (Pharmacia Fine Chemicals, Piscataway, NJ) were added to cells to final concentrations of 10 or 25 μg/ml, respectively, and flow was resumed. Computer analysis for $[Ca^{2+}]_i$ vs. time and percent-responding cells vs. time was performed as previously described (44) using the software Multitime (Phoenix Flow Systems, San Diego, CA).

T Cell Activation. Round-bottom 96-well microtiter plates (Corning Glass Works, Corning, NY) were incubated with either mAb 145-2C11 (37) (10 μg/ml in PBS), mAb H57-597 (38) (1:10,000 dilution (vol/vol) of ascites in PBS, provided by Dr. J. Ledbetter (Oncogene, Seattle, WA), or with PBS alone for 2 h at 37°C and

[1] *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular calcium concentration; hGH, human growth hormone.

then washed five times with PBS. Spleen mononuclear cells (4×10^4–2×10^5) were added to wells in 0.2 ml of CT.4S medium and incubated for 72 h at 37°C in a humidified atmosphere with 5% CO_2. All wells were pulsed for the last 12 h of incubation with 1 μCi of [^{3}H]thymidine. Cells were harvested onto glass fiber filters and assayed for [^{3}H]thymidine incorporation by liquid scintillation counting. All assays were performed in triplicate.

Ig Isotype Analysis. Ig levels were determined by an isotype-specific sandwich ELISA technique. The assays for IgG1, IgG3, and IgE levels were performed as previously described (45). IgG2a, IgG2b, IgA, and IgM levels were similarly determined using appropriate isotype-specific goat anti-mouse antibodies (Southern Biotechnology Associates, Birmingham, AL) as first-step reagents to coat the wells of microtiter plates. After blocking with 5% nonfat dry milk, wells were incubated with plasma or isotype standards (Southern Biotechnology Associates) diluted in PBS/3% BSA for 1 h, washed six times with PBS/0.5% Tween (Sigma Chemical Co., St. Louis, MO), and then incubated with appropriate isotype-specific, horseradish peroxidase–conjugated, goat anti-mouse antibodies (Southern Biotechnology Associates) for 1 h. After six washes with PBS/0.5% Tween, peroxidase-conjugated antibody was detected using TMB Microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) as specified by the manufacturer. Plates were read on an ELISA reader (Dynatech Laboratories, Inc., Alexandria, VA). Data analysis of test samples was performed as previously described (45).

Results

Selective Intrathymic Expression of IL-4 in lck–IL-4 Transgenic Mice. To gain insight into the potential role of IL-4 in T-lineage cell development in vivo, transgenic mice were generated in which increased IL-4 expression was selectively targeted to the thymus. Fig. 1 details the transgene expression construct (*lck*–IL-4) in which a 3.2-kb *lck* proximal promoter segment (24, 25) was placed immediately 5′ to a full-length murine IL-4 cDNA clone. The *lck* proximal promoter has previously been shown to direct the expression of other transgenes in a thymocyte-specific manner (reference 25 and Perlmutter, R. M., unpublished results). The IL-4 cDNA segment was embedded within the hGH gene since intronic sequences appear to augment transgene expression (46). By positioning the cDNA 5′ to the translation initiation codon of the hGH gene, no growth hormone expression is observed (data not shown).

Three transgene-positive mice that expressed detectable transgene-derived mRNA were obtained after injection of the *lck*–IL-4 construct. These founder animals, designated 1315, 4453, and 4475 displayed a distinct perturbation of T-lineage cell development that included (*a*) thymic hypoplasia; (*b*) a marked decrease in the number of $CD4^+CD8^+$ thymocytes; (*c*) an absolute increase in the number of $CD4^-CD8^+$ thymocytes; (*d*) a decreased number of peripheral $CD4^+$ T cells; and (*e*) the virtual absence of peripheral $CD8^+$ T cells. The estimated number of copies of the transgene in the 1315, 4453, and 4475 founders was 8, 5, and 5, respectively, based on densitometry of tail DNA blots hybridized with an IL-4 cDNA probe (data not shown). Since the transgene copy number and the degree of perturbation of T-lineage cells in these three founders were similar, this indicated that the particular integration site of the transgene did not have a significant influence on the phenotype we observed. Described below, in detail, are the results obtained with the 1315 founder and six generations of its progeny.

Tissue expression of the *lck*–IL-4 transgene was determined by hybridization of RNA blots with an IL-4 cDNA probe (Fig. 2). Transgene-encoded IL-4 transcripts, which contain 3′ hGH sequences (Fig. 1), were significantly larger and readily distinguished from the endogenous 0.7-kb IL-4 transcripts found in EL-4 cells. Two major sizes of transgenic IL-4 mRNA were observed, presumably because of alternative splicing of hGH intron sequences. Low levels of transgene transcripts were detected in spleen (~1–2% of that found in thymocytes based on scanning densitometry) as well as LN tissue

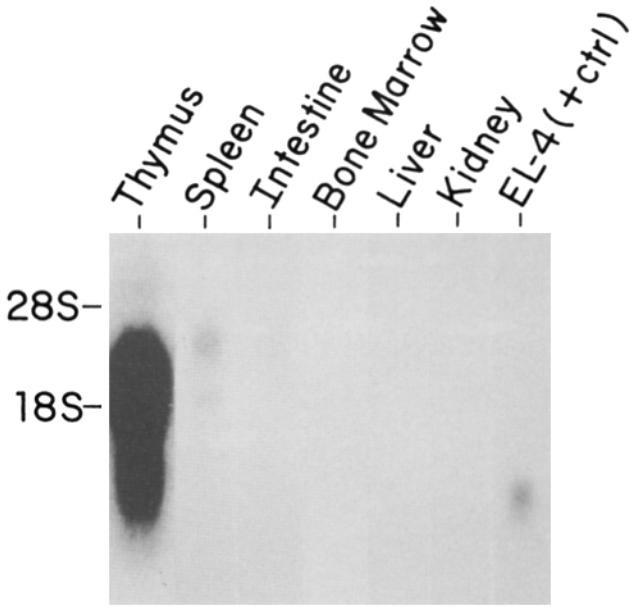

Figure 2. Total RNA from tissues of *lck*–IL-4 mice hybridized with an IL-4 cDNA probe. All lanes were loaded with 10 μg. RNA from PMA-stimulated EL-4 cells, which express endogenous IL-4 gene transcripts, served as a positive control.

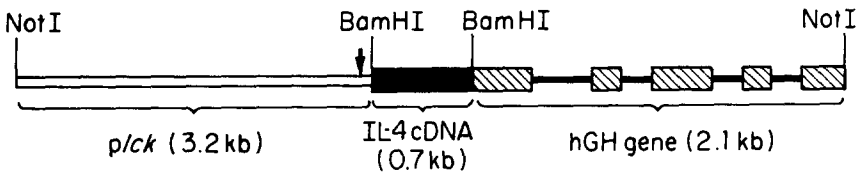

Figure 1. Map of the *lck*–IL-4 transgene construct. The transcription start site of the *lck* promoter is indicated by an arrow. The IL-4 cDNA segment is indicated by the solid rectangle. hGH exon sequences are indicated by crosshatching.

Table 1. *IL-4 Secretion (pg/ml) by Unstimulated Cells from lck–IL-4 and Littermate Control Mice*

Cell Type	$^{-}$11B11 mAb	$^{+}$11B11 mAb
Exp. 1: 6-wk-old mice		
lck–IL-4 thymocytes	29	<10
LM control thymocytes	<10	ND
lck–IL-4 splenocytes	<10	<10
LM control thymocytes	<10	ND
lck–IL-4 bone marrow cells	<10	<10
LM control bone marrow cells	<10	ND
Exp. 2: 9-wk-old mice		
lck–IL-4 thymocytes	69	<10
lck–IL-4 thymocytes	44	<10
LM control thymocytes	<10	ND
Exp. 3: 18-d gestation embryo		
lck–IL-4 thymocytes	28	<10
LM control thymocytes	<10	<10

(data not shown), but not in nonlymphoid tissues. Endogenous IL-4 transcripts were undetectable in RNA from all freshly isolated tissues of transgenic as well as littermate control mice (Fig. 2, data not shown). Taken together, these results demonstrated that *lck*–IL-4 mice had increased expression of IL-4 mRNA which was largely confined to the thymus.

The CT.4S cell proliferation assay (34) was used to measure the amount of biologically active IL-4 secreted by transgenic or control cells after short-term in vitro culture. Increased expression of IL-4 mRNA by thymic tissue in *lck*–IL-4 mice was paralleled by IL-4 secretion by thymocytes beginning as early as day 18 of fetal life (Table 1). Anti–IL-4 mAb completely neutralized this proliferative activity in the CT.4S assay, confirming that it was attributable to IL-4. The amount of IL-4 secreted by unstimulated thymocytes over a 24-h period ranged from ~25 to 70 pg/ml based on the activity of purified rIL-4 in the CT.4S assay. In contrast, IL-4 was not detected in cultures of control thymocytes, or in cultures of splenocytes or bone marrow cells from either transgenic or control mice.

Perturbation of Thymocyte Development. The amount of thymic tissue in *lck*–IL-4 mice was markedly reduced compared with controls, with the loss of a clearly defined cortical region (Table 2, Fig. 3 *A* and *B*). In virtually the entire organ, thymocytes were found in a loosely packed pattern characteristic of the normal medulla (Fig. 3 *C* and *D*). This reduction in thymocyte number compared with controls was evident as early as day 3 after birth but not at day 16.5 of fetal gestation (data not shown). Consistent with these findings, the proportion and absolute number of $CD4^{+}$ $CD8^{+}$ cells, a population which normally includes >75% of thymocytes and is found mainly in the cortex (47), were both markedly lower in transgenic compared with control mice (Fig. 4 *A*, Table 2). This decrease was accompanied by an increased proportion of $CD4^{-}CD8^{-}$, $CD4^{+}CD8^{-}$, and, particularly, $CD4^{-}CD8^{+}$ thymocytes. The $CD4^{-}CD8^{+}$/$CD4^{+}CD8^{-}$ thymocyte ratio in *lck*–IL-4 mice was typically greater than 1:1, and the absolute numbers of $CD4^{-}CD8^{+}$ cells exceeded those found in controls (Table 2). A significant proportion of $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ *lck*–IL-4 thymocytes were also noted to have moderately reduced surface expression of CD4 or CD8, respectively, compared with thymocytes from controls (Fig. 4 *A*).

Table 2. *Abundance of Thymocytes and Thymocyte Subpopulations in lck–IL-4 and Littermate Control Mice*

	Total thymocytes	Surface phenotype (% of cells) thymocytes			
		$CD4^{-}CD8^{-}$	$CD4^{+}CD8^{-}$	$CD4^{-}CD8^{+}$	$CD4^{+}CD8^{+}$
	$\times 10^{6}$				
Exp. 1: 3-wk-old mice					
lck–IL-4	29	8 (2)*	25 (7)	42 (12)	25 (7)
lck–IL-4	31	10 (3)	25 (9)	46 (14)	19 (6)
LM control	151	4 (6)	11 (17)	4 (6)	81 (122)
LM control	139	7 (10)	15 (21)	7 (10)	71 (99)
Exp. 2: 9-wk-old mice					
lck–IL-4, PBS treated	28	9 (3)	28 (8)	33 (9)	30 (8)
lck–IL-4, 11B11 treated	126	11 (14)	24 (30)	13 (16)	53 (67)
lck–IL-4, 11B11 treated	110	9 (10)	24 (26)	12 (13)	55 (60)
LM control, PBS treated	106	4 (4)	8 (8)	3 (3)	85 (90)
LM control, 11B11 treated	88	4 (4)	10 (9)	2 (2)	84 (74)

* The absolute number of cells ($\times 10^{6}$) for each surface phenotype is indicated in parentheses.

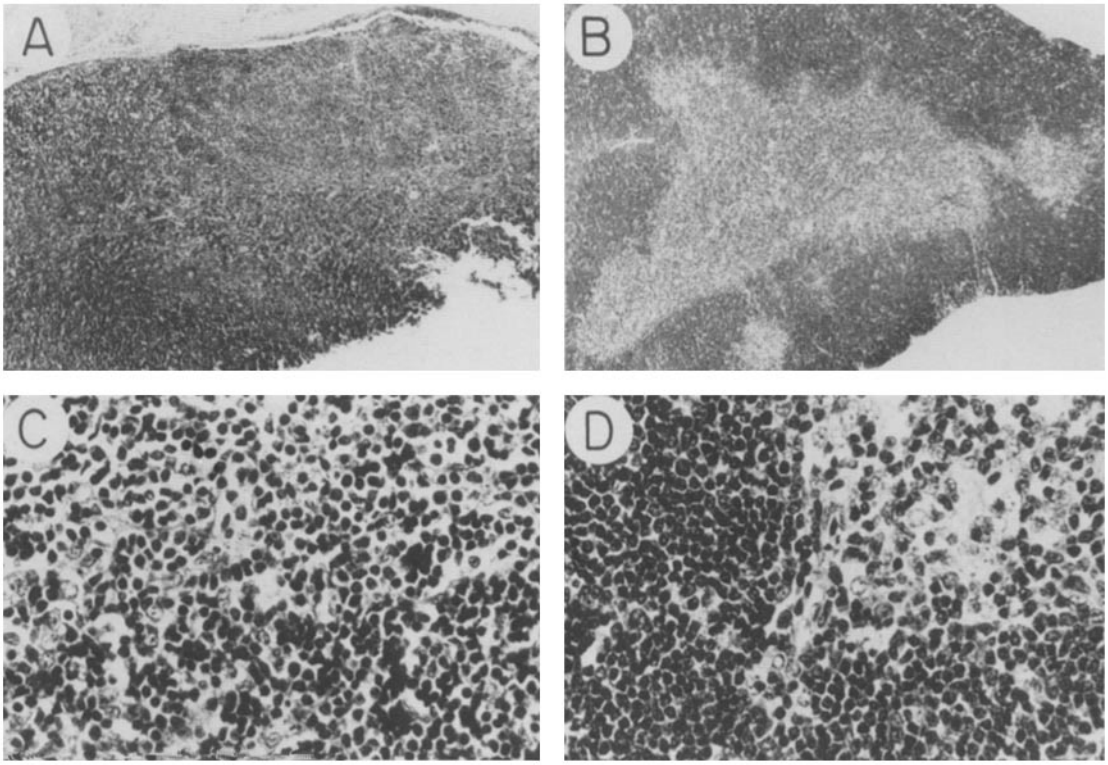

Figure 3. Histologic sections of thymi from *lck*–IL-4 mice or nontransgenic littermate controls after formalin fixation and staining with hematoxylin and eosin. Low-power magnification ×2.5 of a *lck*–IL-4 thymus (*A*) demonstrating markedly decreased size and cellularity and loss of a distinct cortical region compared with a nontransgenic littermate thymus (*B*). Higher-power magnification (×25 of the *lck*–IL-4 thymus (*C*) showing the loss of a distinct cortical–medullary junction, and an overall loose-packing of cells characteristic of the normal medulla. In contrast, the thymus from a nontransgenic littermate (*D*) has a distinct cortical–medullary junction, with the cortical region densely packed with thymocytes; the medullary region is located in the upper right corner of the photomicrograph.

An increased proportion of *lck*–IL-4 thymocytes expressed surface TCR-α/β as assessed by two-color staining with CD3 and pan-α/β TCR mAbs (Fig. 4 *B*), while the small numbers (<5%) of thymocytes bearing TCR-γ/δ did not differ significantly from that found in littermate controls (data not shown). To further characterize the *lck*–IL-4 thymocyte populations that expressed surface TCR, three color staining for CD3, CD4, and CD8 was performed. This revealed that most $CD4^-CD8^+$ *lck*–IL-4 thymocytes expressed detectable surface TCR, although a significant proportion of this population had lower amounts of surface TCR compared with control $CD4^-CD8^+$ thymocytes (Fig. 4 *C*, *left*). Thymocytes with this $CD3^+CD4^-CD8^+$ surface phenotype normally first appear at the very end of fetal ontogeny (day 19) and include $CD4^-CD8^+$ cells destined to emigrate from the thymus (47). In contrast, there was no significant increase in the number of $CD3^-CD4^-CD8^+$ thymocytes, a population which occurs earlier in fetal ontogeny (day 15), and is apparently the direct precursor of $CD3^-CD4^+CD8^+$ thymocytes (48, 49). $CD4^+CD8^-$ thymocytes from transgenic and control mice exhibited similar levels of CD3 expression (data not shown). However, the proportion of $CD4^+CD8^+$ thymocytes that were $CD3^+$ was strikingly increased in *lck*–IL-4 mice compared with controls (Fig. 4 *C*, *left* and *right*). In agreement with previous work (36), the amount of CD3 staining on $CD3^+CD4^+CD8^+$ thymocytes from either transgenic or control animals was relatively low compared with that found on most single-positive cells.

Reversal of Thymic Abnormalities in lck–IL-4 Mice Treated with Anti–IL-4 Antibody. To confirm that IL-4 secretion was responsible for the phenotypic abnormalities of T-lineage cells in *lck*–IL-4 mice, transgenic or control mice were treated for 1 mo with injections of either anti–IL-4 mAb (11B11) or PBS. Transgenic mice that received anti–IL-4 mAb had striking increases in thymocyte number (Table 2) as well as partial normalization of thymic architecture (data not shown). The composition of thymocyte subsets defined by surface expression of CD4, CD8, and CD3 was also modified towards that found in normal mice, after treatment with anti–IL-4 but not PBS (Fig. 4 *C*, Table 2). There were no discernible effects on the thymi of control mice as a result of treatment with either anti–IL-4 or PBS. These results showed that the perturbation of thymic development observed in *lck*–IL-4 mice was critically dependent on the secretion of IL-4.

Peripheral T Cells in lck–IL-4 Mice. Phenotypic alterations in the peripheral T cell compartment were also evident in *lck*–IL-4 mice. Most striking was the virtually complete absence of $CD8^+$ T cells in the LN (Fig. 5 *A*), spleen, and

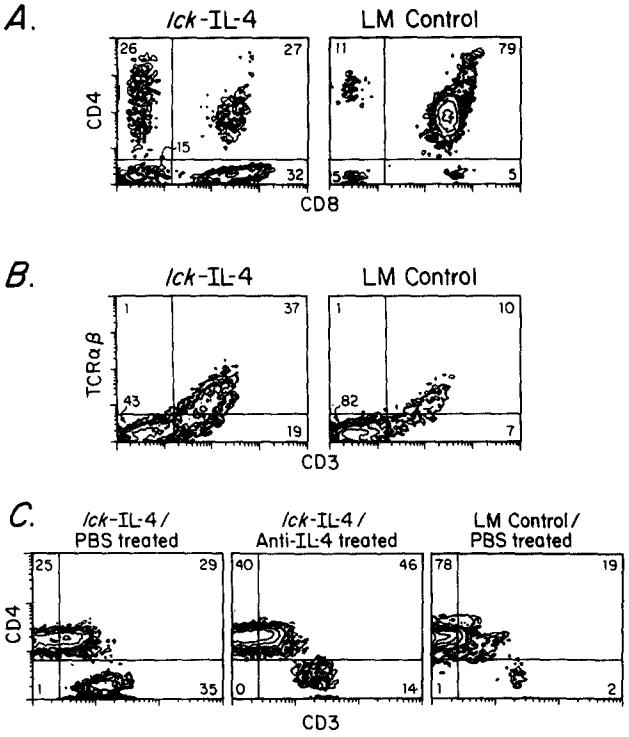

Figure 4. Two- and three-color immunofluorometric analysis of thymocytes from *lck*–IL-4 and nontransgenic littermate (*LM*) control mice. Thymocytes from 6-wk-old mice stained for (*A*) CD4 (GK1.5) vs. CD8 (104.21) and (*B*) TCR-α/β (H57-597) vs. CD3 (145-2C11). (*C*) Three-color analysis of thymocytes from 9-wk-old mice, after in vivo treatment with PBS or anti–IL-4 mAb (11B11), for CD4 (GK1.5) vs. CD3 (500A.2) on electronically gated cells positive for CD8 (104.21).

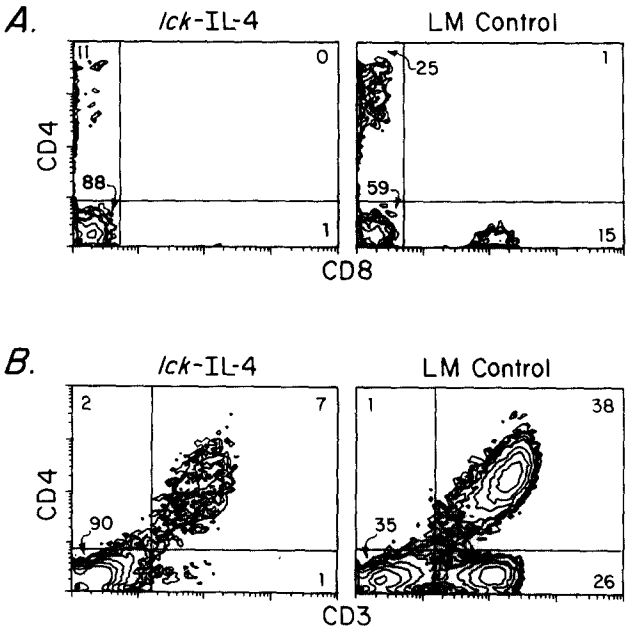

Figure 5. Two-color immunofluorescence analysis of LN cells from *lck*–IL-4 and nontransgenic littermate (*LM*) control mice for (*A*) CD4 (GK1.5) vs. CD8 (104.21) and (*B*) CD4 (GK1.5) vs. CD3 (500A.2).

peripheral blood (data not shown). $CD4^+$ T cells were present at all of these sites, although in significantly reduced numbers (Fig. 5, *A* and *B*). Unlike $CD4^+CD8^-$ thymocytes, the *lck*–IL-4 peripheral $CD4^+$ T cell population had surface levels of CD4 comparable with T cells from controls (Fig. 5 *B*). $CD4^+$ T cells in *lck*–IL-4 mice also displayed normal levels of TCR (Fig. 5 *B*) as well as CD5, CD45, and Thy-1 (data not shown).

Thymocyte and Peripheral T Cell Activation Is Significantly Altered in lck–IL-4 Mice. To determine if the *lck*–IL-4 transgene led to functional as well as phenotypic alterations of T-lineage cells, individual thymocytes and peripheral T cells were analyzed for activation-induced changes in $[Ca^{2+}]_i$. In all experiments, transgenic and control cell populations with analogous patterns of CD4 and CD8 surface expression were directly compared. As shown in Fig. 6, the calcium responses of transgenic thymocyte populations after stimulation were distinct from those observed in control cells: transgenic $CD4^+CD8^+$ thymocytes had strikingly greater increases in mean $[Ca^{2+}]_i$ compared with control $CD4^+CD8^+$ thymocytes after treatment with anti-CD3 mAb (*A*), as well as Con A or anti-TCR-α/β mAb (data not shown). In contrast, the calcium response of single-positive thymocytes to anti-CD3 mAb treatment was dramatically lower than that of control populations (*B* and *C*). $CD4^-CD8^-$ transgenic and control thymocytes both had low levels of response under these conditions (data not shown). When splenic $CD4^+$ T cell populations were similarly analyzed, dramatically lower calcium responses were observed in transgenic cells compared with control cells after either anti-CD3 mAb or Con A treatment (*D*). Similar results were obtained using LN $CD4^+$ T cells (data not shown). For all cell populations analyzed, the maximum percent of cells which gave a significant calcium response paralleled the peak mean $[Ca^{2+}]_i$ achieved by these cells (see legend to Fig. 6). Transgenic peripheral $CD8^+$ T

Table 3. *Ig Isotype and IgG Subclass Levels (μg/ml ± SEM) in Adult lck–IL-4 (n = 12) and Littermate Control (n = 7) mice*

	IgM	IgA	IgE	IgG1	IgG2a	IgG2b	IgG3
lck–IL-4	644 ± 436	1,510 ± 1,850	2.7 ± 0.1	1,010 ± 806	268 ± 142*	395 ± 108	80 ± 60
LM control	384 ± 200	621 ± 484	2.5 ± 0.3	882 ± 898	142 ± 63	330 ± 94	35 ± 16

* $p < 0.05$ compared with LM control using the two-tailed Student's *t* test.

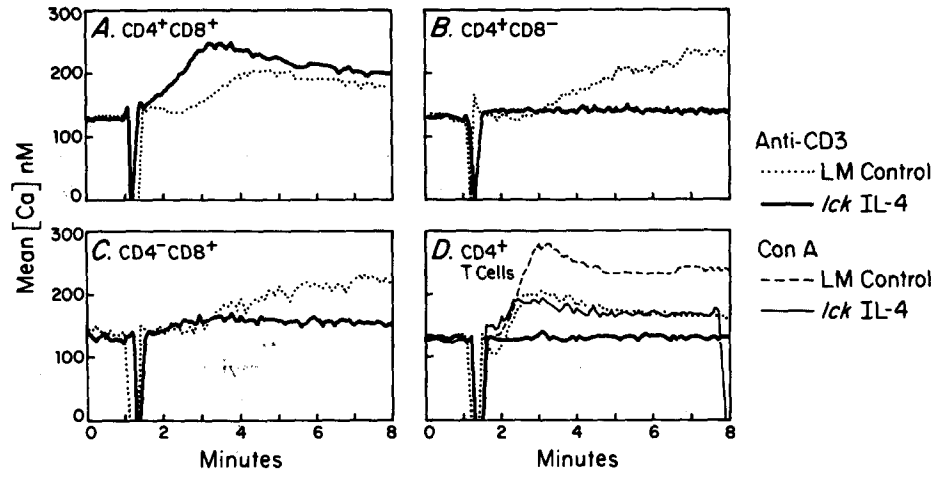

Figure 6. $[Ca^{2+}]_i$ in stimulated thymocytes and splenic $CD4^+$ T cells from *lck*-IL-4 and littermate (*LM*) control mice. As described in Materials and Methods, cells were sequentially loaded with indo-1 dye, stained with PE-conjugated CD8 and FITC-conjugated CD4 mAbs, and analyzed for $[Ca^{2+}]_i$, using flow cytometry to gate on cell populations expressing surface CD4 and/or CD8. (*A*) $CD4^+CD8^+$, (*B*) $CD4^+CD8^-$, and (*C*) $CD4^-CD8^+$ thymocytes stimulated with anti-CD3 mAb (145-2C11). (*D*) Splenic $CD4^+$ T cells stimulated with either anti-CD3 mAb (145-2C11) or Con A. Mean $[Ca^{2+}]_i$ of all cells analyzed vs. time is plotted in each histogram. The maximum percentage of transgenic (*TG*) or littermate control (*LM*) cells which responded with elevations of $[Ca^{2+}]_i$ by the end of the 8-min period was as follows: (*A*) TG, 68%; LM, 55%; (*B*) TG, 12%; LM, 77%; (*C*) TG, 29%; LM, 74%; (*D*) anti-CD3; TG, 13%, LM, 52%; Con A; TG, 47%, LM, 75%.

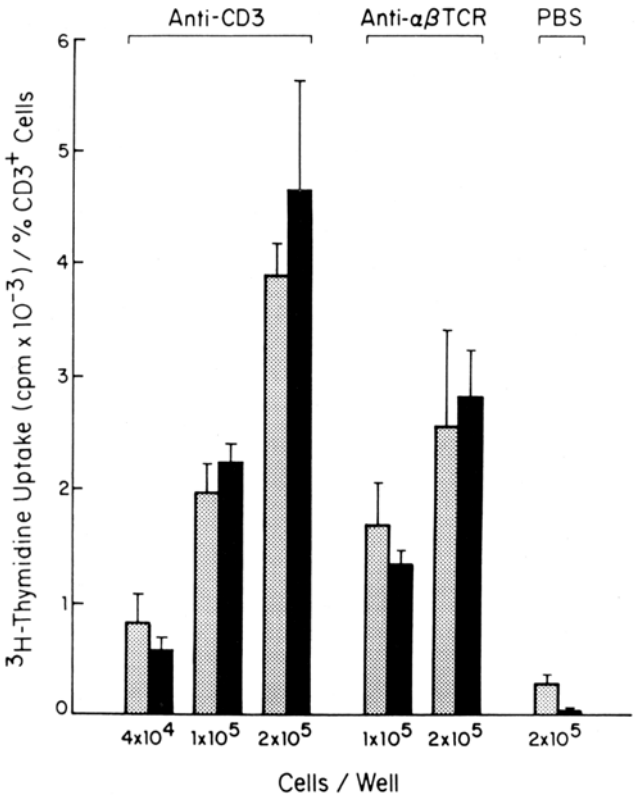

Figure 7. Proliferative response of *lck*-IL-4 and littermate control spleen mononuclear cells to crosslinking with anti-CD3 or anti-TCR-α/β mAbs. After 60 h, cultures were pulsed for 12 h with [^{3}H]thymidine. Counts were calculated as the mean ± SEM of triplicate determinations. *Lck*-IL-4 cell are indicated by stippled bars, and littermate control cells are indicated by solid bars. The data have been normalized with respect to the percentage of $CD3^+$ cells found in the spleen mononuclear cell preparations. In the experiment shown, 16% of *lck*-IL-4 cells and 31% of littermate control cells were surface $CD3^+$. Similar results were obtained in two additional experiments.

cells were not present in sufficient numbers for them to be similarly analyzed.

In contrast to their decreased TCR-mediated fluxes in $[Ca^{2+}]_i$ splenic T cells from *lck*-IL-4 mice appeared to proliferate normally after cross-linking of their TCR-CD3 complexes with either anti-CD3 or anti-pan-α/β TCR mAb (Fig. 7). In addition to their apparently normal proliferative response, *lck*-IL-4 splenic T cells activated by anti-CD3 mAb also accumulated amounts of IL-2 mRNA which were similar to those in littermate control spleen T cells (data not shown).

Additional Abnormalities in lck-IL-4 Mice. *Lck*-IL-4 mice were remarkably susceptible to parasitic infection. Conventionally housed transgenic mice, but not controls, invariably developed severe intestinal infection with the pinworm *Syphacia obvelata* (50). Megacolon, rectal prolapse, and cecal perforation were frequent findings. Most severely infected mice died between 6 and 12 wk of age. In contrast, infection of conventionally housed *lck*-IL-4 mice with mouse hepatitis virus only rarely caused significant disease. Since IL-4 has been shown to increase IgE and IgG1 isotype production by activated B cells in vitro (8), and to be directly involved in promoting IgE production in vivo during parasitic infection (11, 12), we determined the concentrations of these and, for comparison, other Ig isotypes in the circulation of pinworm-infected *lck*-IL-4 mice and littermate controls (Table 3). There were no significant differences between transgenic and control mice in their plasma levels of IgM, IgA, IgE, IgG1, IgG2b, or IgG3. An unexpected finding was that IgG2a levels were significantly higher in infected *lck*-IL-4 mice compared with controls. This observation may reflect the higher antigenic load experienced by *lck*-IL-4 mice. These results, which suggested that B cell function was not grossly perturbed by the IL-4 transgene, were consistent with the normal histologic appearance of lymphoid follicles and the normal numbers of peripheral $B220^+$ B cells found in *lck*-IL-4 mice (data not

shown). Since IL-4 has also been shown to act as a growth factor for mast cells (5), we histologically examined these transgenic mice for increased mast cell numbers using a Giemsa stain. However, we failed to find significant increases in mast cell abundance in any organs of *lck*–IL-4 mice, including the skin (data not shown).

Discussion

IL-4 has previously been shown to promote the growth of isolated thymocytes including fetal and adult $CD4^-CD8^-$ cells, adult $CD4^+CD8^-$ and $CD4^-CD8^+$ cells, but not $CD4^+CD8^+$ cells (15–21). In vitro studies using murine $CD4^-CD8^-$ or human $CD4^-CD8^-CD3^-CD1^-$ thymocytes suggest that IL-4 preferentially promotes differentiation of double-negative or $CD8^+$ γ/δ T-lineage cells (16, 21). Consistent with this interpretation, incubation of isolated double-positive or single-positive TCR-α/β^+ thymocytes with IL-4 does not significantly alter their surface expression of CD4, CD8, or CD3 (Lewis, D., unpublished observations). These results contrast with those obtained in our in vivo model, which demonstrate a profound influence of IL-4 on the abundance, surface phenotype, and TCR-mediated activation of thymocytes, particularly TCR-α/β^+ cells at later stages of maturation, and on the ability of mature thymocytes to successfully colonize the periphery. We conclude that proper interactions between cytokines and the thymic microenvironment are crucially important in regulating multiple aspects of T cell development.

IL-4 expression by normal murine fetal thymocytes is highest at day 15 of gestation, when the predominant thymocyte population is $CD4^-CD8^-$, but rapidly declines by day 16, becoming undetectable from day 18 of gestation onward (13). This suggests a potential physiologic role for IL-4 in early thymic development. In preliminary experiments, we have examined the effects of the *lck*–IL-4 transgene on fetal thymic ontogeny at day 16.5–17 of gestation and have found no significant differences between transgenic and control thymocytes with respect to CD3, CD4, or CD8 surface expression (data not shown). Further, no significant differences were observed in the percentage of thymocytes bearing TCR-γ/δ, or in their surface expression of Thy-1, CD5, or CD45. IL-4 secretion by *lck*–IL-4 but not control thymocytes is detectable as early as day 18 of gestation (Table 1), and it is likely, based on the normal ontogeny of expression of the endogenous proximal *lck* promoter, that thymocytes express the *lck*–IL-4 transgene beginning as early as day 14 of gestation (reference 51; Lewis, D., and R. Perlmutter, unpublished observations). These findings argue that increased intrathymic expression of IL-4 has little discernible impact on T cell development before the appearance of double-positive thymocytes. In contrast, the development of double-positive and TCR-α/β–bearing single-positive thymocytes is dramatically affected by in situ IL-4 production in adult mice. These results suggest that the progressive decrease in IL-4 expression by thymocytes which occurs during late fetal ontogeny may be critical for subsequent normal T cell development.

The thymi of *lck*–IL-4 mice lacked a clearly defined cortex, a region which in normal mice predominantly contains double-positive thymocytes (47). Cortical $CD4^+CD8^+$ thymocyte depletion is also a prominent feature of thymi from mice that have been physiologically stressed or treated with glucocorticoids. However, under these conditions the $CD4^+CD8^-$/$CD4^-CD8^+$ single-positive thymocyte ratio, which normally is ~2–3:1, remains unchanged (52, 53). In contrast, in *lck*–IL-4 mice this ratio was typically 1:1 or less, reflecting an absolute increase in the number of $CD4^-CD8^+$ thymocytes. Hence, the alterations observed in thymic development in *lck*–IL-4 mice, which clearly depended on the secretion of IL-4 (Table 2, Exp. 2; Fig. 4 *C*), are unlikely to be simply the result of systemic stress.

The finding that $CD4^+CD8^+$ thymocytes had increased TCR surface expression in *lck*–IL-4 mice is particularly noteworthy, since no cytokine has previously been shown to mediate this effect. Although the mechanism by which IL-4 increases surface TCR expression on double-positive cells remains to be determined, the results of treatment of *lck*–IL-4 mice with anti–IL-4 mAb clearly show that this effect is dependent on the actual secretion of IL-4. Similar increases in surface TCR expression by double-positive thymocytes have also been observed after in vivo treatment of normal mice with anti-CD4 but not anti-CD8 mAb (54, 55), or when normal thymocytes are incubated in vitro in suspension but not as tissue fragments (55, 56). In both cases, a posttranslational mechanism, in which assembled CD3-TCR complexes are more efficiently transported from the endoplasmic reticulum to the cell surface, appears to be involved (55). These findings have led to the proposal that the thymic microenvironment may provide a signal through the CD4 molecule of $CD4^+CD8^+$ thymocytes which attenuates the surface expression of TCR by these cells (55). We have found that the inclusion of IL-4 in suspension cultures of normal thymocytes does not further augment surface TCR expression by double-positive thymocytes (Lewis, D., unpublished observations), suggesting that, like anti-CD4 mAb treatment, an intact thymic microenvironment is required for this IL-4–mediated effect. It remains to be determined whether an altered CD4-mediated signal in *lck*–IL-4 double-positive thymocytes is also responsible for their increased TCR expression, or whether thymi from *lck*–IL-4 mice have decreased surface expression of class II MHC, the presumed physiologic surface ligand for CD4 within the thymus (57).

An increasing body of evidence indicates that engagement of TCR-α/β on $CD4^+CD8^+$ thymocytes by self-MHC, presumably as part of a complex with self-antigen peptides, is critical both for the development of T cells with receptors capable of recognizing foreign antigen–self-MHC complexes (positive selection) as well as the deletion of T cells bearing TCR with potentially harmful self-reactivity (negative selection; for review see reference 58). The signals by which TCR engagement leads to either positive or negative selection of thymocytes remain poorly understood. In a model for negative selection using cultured fetal thymus lobes, $CD4^+CD8^+$ thymocytes which mobilize calcium after treat-

ment with anti-CD3 or anti-TCR-α/β mAbs are also eliminated by these mAbs during organ culture (59–61). This correlation between TCR-mediated calcium fluxes and in vitro deletion also provides a plausible explanation for the depletion of $CD4^+CD8^+$ thymocytes observed in all lines of *lck*–IL-4 mice. *Lck*–IL-4 $CD4^+CD8^+$ thymocytes, by virtue of their increased mobilization of $[Ca^{2+}]_i$ after TCR–CD3 complex engagement, might also be more prone to intrathymic deletion than normal double-positive thymocytes.

The expression of IL-4 intrathymically exerted different effects on mature $CD4^+CD8^-$ as compared with $CD4^-CD8^+$ T-lineage cells. Only $CD4^-CD8^+$ thymocytes were increased in absolute numbers in these thymi, while, in contrast, only $CD4^+$ and not $CD8^+$ T cells were found in the peripheral compartment. This suggests that $CD4^-CD8^+$ thymocyte emigration was blocked in *lck*–IL-4 mice, or that if emigration occurred, these cells failed to proliferate at peripheral sites. A significant, but less severe, decrease in the emigration of $CD4^+CD8^-$ thymocytes might also account for the reduced numbers of peripheral *lck*–IL-4 $CD4^+$ T cells we observed. Although the mechanisms involved in the emigration of single-positive thymocytes into the circulation are poorly understood, our results suggest that cytokines such as IL-4 can differentially regulate this process in $CD4^+CD8^-$ and $CD4^-CD8^+$ T lineage cells. The observation that IL-4 in vitro is significantly more effective in promoting the growth of $CD4^-CD8^+$ compared with $CD4^+CD8^-$ thymocytes (17) indicates that these two populations may differ intrinsically in their responses to IL-4 by other criteria as well.

Peripheral T cells as well as mature TCR-α/β–bearing single-positive thymocytes from *lck*–IL-4 mice were markedly defective in their mobilization of calcium in response to anti-CD3 mAb or the mitogen, Con A (Fig. 6). However, *lck*–IL-4 T cells appeared to proliferate as well as control cells in response to anti-CD3 or anti-TCR-α/β mAb (Fig. 7). Although thymidine incorporation by *lck*–IL-4 splenocytes was lower than that by splenocytes from littermate controls, this decrease was proportional to the lower percentage of peripheral T cells found in this transgenic population (see legend to Fig. 7). The mechanism(s) responsible for this relatively selective defect in proximal signal transduction by mature *lck*–IL-4 T-lineage cells remain to be defined. T cell proliferation after activation via the TCR–CD3 complex has previously been shown to be dependent on the interaction of IL-2 with its specific high-affinity receptor (62), suggesting that this interaction occurred normally in *lck*–IL-4 peripheral T cells. This interpretation is supported by the fact that IL-2 mRNA accumulation in *lck*– IL-4 cells activated with either anti-CD3 mAb, Con A, or ionomycin and PMA was at least equal to that observed in littermate controls (data not shown). Therefore, it is possible that *lck*–IL-4 and peripheral T cells may be capable of IL-2 production by a calcium-independent pathway. Since a calcium-independent pathway for IL-2 production has previously only been described in murine T cell hybridoma line variants or mutants deficient in CD3-η chains (63, 64), it will be of interest to confirm that TCR-mediated proliferation of mature *lck*–IL-4 T-lineage cells is calcium independent and, if so, whether these cells have decreased CD3-η expression as well as reduced activation-induced phosphoinsoitide hydrolysis. Alternatively, mature *lck*–IL-4 thymocytes and T cells may be more susceptible to inhibition of TCR-mediated calcium mobilization by anti-CD4 or -CD8 mAb, which were used to identify cell subsets in these assays, than are control cells. Such inhibition of TCR-mediated calcium mobilization by anti-CD4 mAb has previously been observed in certain T cell clones (65).

An unexpected finding in *lck*–IL-4 mice was their susceptibility to severe infection by the pinworm *Syphacia obvelata*. Previous adoptive transfer studies with nude mice have indicated that T-lineage cells may be critical for the control of this pathogen (50). In other murine models, $CD4^+$ T cells have also been shown to be important for the control of other parasitic infections, including *Nippostrongylus* and *Leishmania* (66, 67). Therefore, it is possible that the generally low numbers of peripheral *lck*–IL-4 T cells, as well as their apparent functional defects, could both contribute to the vulnerability of *lck*–IL-4 mice to parasitic infection. It remains to be shown that *lck*–IL-4 T cells have defective antigen-specific responses in vitro or in vivo.

While preparing this manuscript we became aware of data by Tepper et al. (68) showing that an IL-4 transgene under the control of the μ H chain enhancer results in the increased expression of IL-4 by T- as well as B-lineage cells. These mice demonstrate most of the phenotypic alterations in T-lineage cells we have observed in *lck*–IL-4 mice, confirming that these results are attributable to the IL-4 gene product itself. However μ H chain enhancer–IL-4 transgenic mice differ from *lck*–IL-4 mice in that their serum levels of IgG1 and IgE isotypes are increased, and their skin and eyes are infiltrated with large numbers of mast cells. Our failure to observe these effects on non-T-lineage cells in *lck*–IL-4 mice, despite their constitutive intrathymic production of significant amounts of IL-4, indicates that this cytokine acts locally rather than systemically, even when it is overexpressed in vivo. A soluble form of the IL-4R has recently been identified in the circulation of mice (69, 70). Soluble IL-4R could potentially bind excess IL-4 produced in tissues and prevent the occurrence of systemic effects. Recent studies by Maliszewski et al. (45) have shown that soluble IL-4R can inhibit IL-4–mediated effects in vitro. Our results suggest that mechanisms of this type, limiting the effects of IL-4 at a distance, are of considerable importance. In the absence of such regulation, peripheral IL-4 production in response to inflammatory stimuli could potentially compromise intrathymic T cell development. Hence, it will be of interest to determine whether the levels of circulating IL-4–IL-4R complexes are increased in *lck*–IL-4 mice, and whether the production of soluble receptor is upregulated in response to the constitutive synthesis of IL-4 intrathymically.

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